Evaluation and Comparison of Immunchromatography and Immunofluorescent Techniques in Diagnosis and Epidemiological Studies in Iraqi Kala-Azar.

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ABSTRACT:

BACKGROUND:

Infantile kala-azar is an endemic protozoal disease prevalent among children in Iraq. A rapid diagnostic laboratory tests are required for immediate treatment.

OBJECTIVE:

A prospective study was conducted in two pediatric hospitals in Baghdad during the period from Oct. 2005 to Sept. 2006 to evaluate the efficiency of immunochromatography strip test (IC) with rK39 antigen compared to indirect fluorescent antibody test (IFAT) for serodiagnosis of visceral leishmaniasis (VL) to investigate its use for epidemiological studies in Iraqi kala-azar.

PATIENTSAND METHODS:

The study included 54 proved cases for *Leishmania donovani* (L.D.) bodies in bone marrow, 108 clinically diagnosed cases, 38 with diseases other than VL (tuberculosis, acute amoebic dysentery, urinary schistosomiasis, brucellosis, toxoplasmosis and malaria) and 24 healthy controls. In addition of 3000 patients presented with fever, anaemia and hepatosplenomegaly.

RESULT:

The highest sensitivity of the sera was obtained by IC (92.6%) and by IFAT (96.3%), and the highest specificity by IC (100%) and by IFAT (86.8%). In the epidemiological study with rK39 strip test 66.1% positive reactions were obtained in patients with fever, anaemia and hepatosplenomegaly.

CONCLUSION:

IC strip test with rK39 antigen was more easy to perform but less sensitive than IFAT and the former was more specific than the latter.

KEY WORDS: serological tests , kala-azar, epidemiology .

INTRODUCTION:

Leishmaniasis caused by the haemoflagellate of the genus *Leishmania* is world wide in distribution with major public health importance and considerable impact on its morbidity rate. Leishmaniasis is endemic in the tropical and subtropical regions of 88 countries, 21 in the new world and 67 in the old world, including Africa, America, Asia and Europe accounting for 80,000 deaths per year [1]. Visceral leishmaniasis (VL) which form 500,000 new cases occur annually, 90% are in five countries including Bangladesh, India, Brazil, Nepal and Sudan [1]. In Iraq, VL is endemic and usually detected in infants and children [2-5] and rarely occurs in adults [6]. The present study is planned to investigate the evaluation of rapid immuno chromatographic (IC) technique in diagnosis and serological survey of VL in children compared to indirect fluorescent antibody test (IFAT) and its applicability for epidemiological studies in Iraqi kala-azar.

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MATERIALS AND METHODS:

Blood samples were collected from 224 children attending Al-Ilwyia children hospital and Ibn-Al-Balady maternity and children hospital in Baghdad. The patients were categorized in the following groups:

Group (I): Confirmed VL cases: Blood samples for IC and IFAT were collected from 54 hospitalized children below 5 years of age whom they had positive bone marrow smears for L.D. bodies.

Group (II): IC and IFAT were applied to test 108 blood specimens collected from children below 5 years of age and clinically suspected as having kala-azar in an endemic area of Al-Suwaira district, governorate of Wasit South of Baghdad.

Group (III): Blood samples were collected from 24 children below 5 years of age from different primary health centers with no history of living in

an endemic area with VL and whom were completely healthy by physical examination. This group constituted as a control group.

Group (IV): Infection control with diseases other than VL: Blood samples were collected from 38 children with proven parasitic or bacterial diseases other than VL, these were:

- (12) patients with tuberculosis (sputum positive for acid fast bacilli).
- (7) patients with acute amoebic dysentery (presence of *Entamoeba histolytica* trophozoites in stool).
- (6) patients with urinary schistosomiasis (ova of *Schistosoma haematobium* in urine).
- (6) patients with brucellosis (positive IFAT and Rose-Bengal test).
- (5) patients with toxoplasmosis (positive IFAT).
- (2) patients with malaria (blood smear positive for *Plasmodium vivax*).

In addition, for epidemiological study, a survey was carried out between Oct. 2005 through Sept. 2006 on 3000 patients living in different regions of Iraq presented with fever, anaemia and hepatosplenomegaly, blood samples were tested by IC strip for VL which were distributed to all district hospitals in the governorates included in the present study.

METHODS:

Bone marrow samples were aspirated from 82 hospitalized children whom they were clinically suspected with VL. Smears were stained with Leishman's stain and examined microscopically for L.D bodies.

Immunochromatography (IC): A rapid test kit for the detection of specific antibodies to VL in human serum was applied for a qualitative detection of antibodies to a recombinant antigen (rK39) in human serum. Positive result for VL was indicated when a control and test lines were seen. Only single control line indicates a negative result.

Indirect Fluorescent Antibody Test (IFAT) : Prepared smears of *Leishmania donovani* covering the reaction area of a BIOCHIP slide were used. All the patient sera and controls were tested in a dilution of 1/160. Positive reaction was indicated by the presence of apple-green fluorescence on the promastigote form of the parasite. A negative test show no fluorescence. A positive and negative controls were included in each run.

RESULTS:

To assess different techniques applied for diagnosis of VL, different study groups of children were included in this work to determine the performance indices of available tests namely IFAT and immunochromatographic strip test (IC).

The results obtained by examination of sera from 54 hospitalized patients (out of 82) parasitologically proven cases of VL by IFAT picked up 52(96.3%) of the cases while IC using rK39 recombinant antigen picked up 50(92.6%) of the cases and failed to detect 4 of them (Table-1). By inspecting the data presented in this table it can be said that no positive reactions obtained in sera from healthy control children either by IFAT or IC. IFAT and IC were applied to test 108 sera collected from children clinically suspected as having VL in an endemic area with the disease. These tests picked up 104(96.3%) and 98(90.7%) from those children respectively (Table-2).

Regarding cross reactivity of VL antigen with sera from patients infected with diseases other than VL (tuberculosis, brucellosis, acute amoebic dysentery, urinary schistosomiasis, toxoplasmosis and malaria) is demonstrated in (Table-3). From the data presented in this table it can be seen that no cross reactions were obtained by using the IC test, while in IFAT, cross reaction occurred in 3 cases with tuberculosis and 1 case each in urinary schistosomiasis and brucellosis.

On the other hand, in an epidemiological study of VL by IC strip test with rK39 antigen strip test in 3000 patients with fever, anaemia and hepatosplenomegaly attending hospitals in fifteen governorate during one year period of the study from Oct. 2005 to Sept. 2006; it was found that 1982 (66.1%) were positive. The geographical distribution showed that VL cases were most prevalent in the middle and southern parts of Iraq ; 892 cases (45.0%) were from the middle part of the country (Al-Najaf, Kerbala, Babil, Qadissiya and Wasit), and Wasit was found to be highly endemic. In addition, 776 cases (39.2%) were found in southern parts of the country (Basrah, Missan, Thiqar and Al-Muthana). Lower prevalence was recorded both in the central and northern provinces of the country, where 244 cases (12.3%) were recorded from (Baghdad, Diyala, Salah Al-Din and Anbar), and only 70 cases (3.5%) were recorded from Karkook and Ninewa in the north (Table -4).

From the results obtained it was found that IC was less sensitive than IFAT and the former was more specific than the latter (Table – 5).

DISCUSSION:

Visceral leishmaniasis has been identified in Iraq for more than eight decades causing a serious public health problem with a high risk of

morbidity, mortality and economical costs. In view of high mortality and good response to treatment there is a real need for early detection of VL. The need for immune diagnostic tests varies with each infection, but it is of significance in those infections that cannot be parasitologically diagnosed readily. Immunoassay are also required for those worldwide highly prevalent infections with severe morbidity and mortality, to be used in seroepidemiology and in follow up evaluation of a control program and leishmaniasis is one of these important diseases. In the present study IFAT and IC were evaluated for their applicability in detection of VL cases and also in epidemiological studies of the disease. The results obtained confirmed the usefulness of IFAT and IC techniques to diagnose VL cases and each test has its own advantages. Sensitivity of these tests were assessed with 54 parasitologically proven VL cases (positive bone marrow Leishman stained smears for L.D bodies). IC was less sensitive (92.6%) and picked up 50 of positive bone marrow smears for VL compared to IFAT (96.3%) that picked up 52 of the parasitologically proven cases of VL. Failure of IC to detect four of the parasitologically proven cases is possibly due to low level of the specific antibodies in the early course of the disease. The reason of failure of detection of low antibody level is possibly associated with the lack of amplification effect of enzyme immunoassay whereby the conversion of many molecules of a substrate by a single molecule of enzyme increases the detectability of enzyme labeled antibody molecules [7]. This work is in consistent with the study of Badaro *et al.* [8] who found that sera from early healing infected subjects reacted with *Leishmania* lysate were generally non-reactive with rK39 antigen. When the present finding compared with previous studies it showed some variation, sensitivities of IC strip with rK39 antigen range from 67 to 100%. Patients from India and Nepal show higher sensitivities 100% compared to Brazil 90% and Venezuela 88% and this test was least sensitive in patients from Sudan [9]. Regional variation of results of the rK39 antigen strip test could be explained by the following.

- Differences in the test accuracy between subspecies of *L. donovani* complex as a result of variation in the recombinant antigen [10].
- Genetic differences in the individual patients or in racial subgroups, similar differences in host response in patients exposed to *L. chagasi* in which only 95% of the patients develop progressive disease [11].
- Age factor affecting the level of antibody response may explain the regional differences, Indian kala-azar occurs among individual of all ages while in other parts of the world such as Iraq and Iran where VL is primarily of Mediterranean infantile type [2,3,12].

rK39 antigen is a recombinant product of 39 amino acid repeats encoded by a kinesin gene of VL species. These repeats are conserved between *L. chagasi*, *L. donovani* and *L. infantum*. In the present study, IC was found very specific and sensitive technique. It detected (92.6%) of active VL cases and was 100% specific. These findings correspond to that obtained by Alborzi *et al.* [12] which indicate that rK39 epitope is conserved in Iraqi strains of *L. donovani* and the detectable level of rK39 antibodies in VL patients suggest its reliable application for rapid sensitive and specific serodiagnosis of VL in symptomatic cases living in remote areas where there is poor accessibility to health services.

On the other hand, as with sensitivities of IFAT and IC, their specificities also assessed with 54 parasitologically proven VL cases, the IC was more specific (100%) compared to IFAT (86.8%), because the greater the specificity of a test the fewer the number of false positive results [13]. No false positive results were obtained by IC technique, and there is no cross reactions of VL sera with those from patients with other infectious parasitic or bacterial diseases. The results obtained were in consistent with those of other workers [12,14,15]. When the whole parasite was applied as antigen in commercial IFAT kit, the results showed that false positive reactions occurred with sera from patients with tuberculosis, urinary schistosomiasis and brucellosis. A study by Kar [16] showed that cross reaction occurred with sera from patients with tuberculosis. In another study by Salih [17] that reported the presence of cross reactions of VL sera with those from patients infected with tuberculosis, brucellosis, salmonellosis, toxoplasmosis and malaria. In addition, IFAT technique showed higher cross reactivity (13.2%) with sera from healthy children living in endemic area with VL (Al-Suwaira district, governorate of Wasit), whereas IC strip test gave no cross reactions with the same sera. These results could be due to the presence of antibodies in healthy control subjects from endemic region and this is probably associated with previous exposure of those children to bite of infected sandflies but the infectious dose was not

enough to cause the disease [18]. In addition the bone marrow examination is not a practical method and give high negative results and has no diagnostic value in the field [19]. In the present study, bone marrow stained smears (28 out of 82) of clinically suspected VL cases were negative, probably because the amastigotes were too scanty to be detected in the stained smear.

Regarding the geographical distribution, the disease was most prevalent in the middle and southern regions of Iraq. The majority of diagnosed VL cases came from rural areas with high humidity of soil associated with the presence of several rivers, interconnected canal and dense vegetation. These factors probably provide a suitable environment for breeding of sandflies. High number of stray dogs present in these areas especially in rural areas, may act as reservoir hosts for VL infection. High numbers of malnourished children found in these regions contribute as a predisposing factor for VL [20].

Table 1: A comparison between IFAT and IC with rK39 antigen in diagnosis of parasitologically proved VL cases and healthy controls.

Test	Confirmed VL patients (n = 54)		Healthy children control (n = 24)	
	No.	%	No.	%
IFAT with whole parasite				
Positive	52	96.3	0	0
Negative	2	3.7	24	100
IC with rK39 antigen				
Positive	50	92.6	0	0
Negative	4	7.4	24	100

Table 2 :Evaluation of two serodiagnostic tests (IFAT & IC) in diagnosis of clinically suspected VL cases and controls from infections other than VL.

Test	VL patients (n=108)		Other infection control (n=38)	Cross reactivity %
	No.	%		
IFAT				
Positive	104	96.3	5	13.2
Negative	4	3.7	33	86.8
IC				
Positive	98	90.7	0	0
Negative	10	9.3	38	0

Table 3 :Cross reaction of two serological tests (IFAT and IC) with sera of patients with other infectious diseases.

Non VL patients	No. of sera tested	IFAT	IC
Tuberculosis	12	3	0
Acute amoebic dysentery	7	0	0
Urinary schistosomiasis	6	1	0
Brucellosis	6	1	0
Toxoplasmosis	5	0	0
Malaria	2	0	0
Total	38	5	0

Table 4 :Geographical distribution of 1982 positive VL cases diagnosed by IC strip test in different regions of Iraq.

Locality	No. of children screened	No. of positive VL cases	% of positive VL cases
Middle (Al-Najaf, Kerabala, Babil, Gadissiya, Wassit)	1350	892	45.0
Southern (Basrah, Missan, Thiqar, Al-Muthana)	176	776	39.2
Central (Baghdad, Diyala, Salah-Aldin, Anbar)	369	244	12.3
Northern (Karkook, Ninewa)	105	70	3.5
Total	3000	1982	100

Table 5 :Sensitivity and specificity of two serodiagnostic tests (IFAT, IC) in Iraqi visceral leishmaniasis patients.

Test	IFAT	IC
Sensitivity	96.3%	92.6%
Specificity	71.4%	100%
Positive predictive value	86.72	100
Negative predictive value	90.9	87.5

CONCLUSION:

IC strip test with rK39 antigen was more easy to perform but less sensitive than IFAT and the former was more specific than the latter.

REFERENCES:

1. World Health Organization. Life in twenty first century: a vision for all. World Health Report, World Health Organization Geneva, Switzerland, 1998.
2. Taj-Eldin, S., Al-Hassani, M.H. Kala-azar in Iraq: analysis of 100 cases. Journal of Faculty of Medicine, Baghdad. 1961, 3: 1-9.
3. Halawani, A., Guirges, S.Y. Studies on the epidemiology of infantile kala-azar in Iraq with special reference to leishmanin test. Journal of Egyptian Public Health Association. 1973, 48: 369-383.
4. Nouri, L., Al-Jeboori, T.I. Kala-azar in Iraq. An epidemiological and clinical study. Journal of Faculty of Medicine, Baghdad, 15: 72-85.
5. Alwan, S.J. The present status of visceral leishmaniasis in Baghdad City, M.Sc. Thesis, College of Medicine, University of Baghdad, 1985.
6. Rassam, S.W. Al-Jeboori, T.I. Kala-azar occurrence in an adult. Journal of Faculty of Medicine, Baghdad, 1973, 15: 87-90.
7. Jackson, T.M., Ekins, R.P. Theoretical limitations on immunoassay sensitivity. Journal of Immunological Methods. 1986, 87: 13-30.
8. Badaro, R., Benson, D., Eulalino, M.C. *et al.* rK39 : a cloned antigen of *Leishmania chagasi* that predicts active visceral leishmaniasis. Journal of Infections Diseases. 1996, 173: 758-761.
9. Silvo, F., Regnaldo, D. Performance of recombinant K39 antigen in the diagnosis of Brazilian visceral leishmaniasis. American Journal of Tropical Medicine and Hygiene. 2003, 68: 321-324.
10. Jensen, A.I. *et al.*, Serodiagnosis of *L. donovani* infections: assessment of enzyme – linked immosorbent assay using recombinant *L. donovani* gene B protein (GBP) of a peptide sequence of *L. donovani* GBP. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1999, 93: 157-160.
11. D'Oliveira – Junior, A., Costa, S.R., Carvalko, E.M. Asymptomatic *Leishmania chagasi* infection in relatives and neighbours of patients with visceral leishmaniasis. Memoir Instite Oswaldo Cruz. 1997, 92: 15-20.
12. Alborzi, A., Rasouli, M., Nademi, Z., Kadivar, M.R., Pourabbas, B. Evaluation of rK39 strip test for the diagnosis of visceral leishmaniasis in infants. Eastern Mediterranean Health Journal. 2006, 12: 294-299.
13. Vandepitte, J., Engback, K., Piot, P., Heuck, C.C. Basic Laboratory Methods in Clinical Bacteriology. World Health Organization , Geneva. 2003.
14. Zijlstra, E., Nur, Y., Dejeux, P., Khalil, E.A., El-Hassan, A.M., Green, I. Diagnosing visceral leishmaniasis with recombinant K39 strip test: experience from the Sudan. Tropical Medicine and International Health. 2001, 6: 108-113.

15. Kumar, R., Pai, K., Pathak, K., Sundar, S. Enzyme-linked immunosorbent assay for recombinant K39 antigen in diagnosis and prognosis of Sudian visceral leishmaniasis. Clinical Diagnosis Laboratory and Immunology. 2001, 8: 1220-1224.

16. Kar, K. Serodiagnosis of leishmaniasis. Critical Review in Microbiology. 1995, 21: 123-152.

17. Salih, S.M. Diagnostic, Epidemiological and evaluation of some immunological aspects in patients with visceral leishmaniasis in some region of Iraq. Ph.D. Thesis, College of Science, University of Baghdad, 2006.

18. Sundar, S., Rai, M. Laboratory diagnosis of visceral leishmaniasis. Clinical Diagnosis Laboratory and Immunology. 2002, 9: 951-958.

19. Cahill, K.M. Field techniques in the diagnosis of kala-azar. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1980, 64: 107-110.

20. Marsden, P.D. Leishmaniasis. British Medical Journal. 1990, 300: 666-669.